# Preliminary evidence of *reductive stress* in human cytotoxic T-cells following exercise

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**Abstract**

This study investigated immunophenotypic differences in intracellular thiol redox state of peripheral blood mononuclear cells (PBMCs) isolated from trained (TR, n=9, mean ± SD: age 28 ± 5 years; BMI 23.2 ± 2.6 kg·m$^2$; $\dot{V}O_{2max}$ 56.9 ± 6.1 ml·kg$^{-1}$·min$^{-1}$) and recreationally active (RA, n=11, mean ± SD: age 27 ± 6 years; BMI 24.2 ± 3.7 kg·m$^2$; $\dot{V}O_{2max}$ 45.1 ± 6.4 ml·kg$^{-1}$·min$^{-1}$) participants before and after a maximal aerobic exercise tolerance test. Blood samples were taken before (PRE), during (sample acquired at 70% $HR_{max}$), immediately (POST+0) and 15 minutes post-exercise (POST+15). PBMCs were isolated and reduced thiol analysis (fluorescein-5 maleimide (F5M)) by immunophenotype (CD3+, CD4$^+$ and CD8$^+$) was performed using flow cytometry. A significant increase in cellular F5M fluorescence was observed in CD3$^+$ T-cells at POST+0, with changes driven to a greater extent by CD8$^+$ T-cells (fold change in both groups CD4: +2.3, CD8: +2.8; $p<0.05$). Further analysis revealed a population of highly reduced CD8$^+$ T-cells (*CD8$^+$T-reduced$^+$*) that significantly increased from PRE to POST+0 in RA participants only (RA: +272 cell/µL, $p<0.05$). To further understand these results, *CD8$^+$T-reduced$^+$* and *CD8$^+$T-reduced$^-$* cells were analysed for immunophenotype in response to the same exercise protocol (n=6, mean ± SD: age 24 ± 5 years; BMI 25.7 ± 4.1 kg·m$^{-2}$; $\dot{V}O_{2max}$ 41.33 ± 7.63 ml·kg$^{-1}$·min$^{-1}$). *CD8$^+$T-reduced$^+$* had significantly less lymphoid homing potential (CCR7) POST+0 compared to PRE. This study is the first to demonstrate that lymphocyte populations become more *reductive* in response to acute exercise.

**New & Noteworthy**

The study presented provides the first evidence to suggest that cytotoxic T-cells become transiently reductive in healthy individuals following a single bout of cycling. Detection of these cells was enabled via the use of a flow cytometric assay that incorporates the thiol reactive probe Fluorescein-5 maleimide. Using this method, transient *reductive stress* in viable T-cells is permissible and provides the basis for further research in the area of exercise immunology.

**Abbreviations**

ANOVA: Analysis of Variance, BMI: Body mass index, CV: Coefficient of variance, CD: Cluster of differentiation, CCR7: Chemokine receptor type 7, DTNB: 5,5'-dithiobis (2-nitrobenzoic) acid, EDTA: ethylene diaminetetraacetic acid, FCS: Fetal Calf Serum, F5M: Fluorescein-5 maleimide, $HR_{max}$: Maximum heart rate, GSH: Glutathione, GSSG: Glutathione disulphide, GTP: Guanidine triphosphate, HBSS: Hanks Balance Salt Solution, $H_2O_2$: Hydrogen peroxide, IL: Interleukin, IPAQ: International Physical Activity Questionnaire, NAC: N-acetylcysteine, NADPH: Nicotinamide adenine dinucleotide phosphate, NEM: N-ethylmaleimide, PD-1: Programmed death receptor-1, PBMC: Peripheral Blood Mononuclear Cell, PBS: Phosphate buffered saline, PRDX: Peroxiredoxin, Pre: Blood sample taken before exercise, Post+0: Blood sample taken immediately post-exercise, Post+15: Blood sample taken 15 minutes post-exercise, RONS: Reactive oxygen and nitrogen species, RPM: repetitions per minute, RPMI: Roswell Park Memorial Institute, SD: Standard deviation, -SH: Sulphur-hydryl, TNB: 5-thio-2-nitrobenzoic acid, TR: Trained, VPA: Vigorous physical activity, $VCO_2$: Carbon dioxide consumption, $\dot{V}O_2$: Oxygen uptake, $\dot{V}O_{2\,MAX}$: Maximum oxygen uptake.

**Introduction**

Alterations in peripheral blood immune cell subpopulations during exercise are well characterised, with transient increases in effector $CD8^+$ T-cells and Natural Killer cells

observed at the expense of $CD4^+$ T-cells and B lymphocytes (4). However, the precise mechanisms driving mobilization and tissue extravasation of these cell types are unclear. Studies have demonstrated that global protein oxidation increases after acute exercise, with redox enzyme activity and protein content (i.e. superoxide dismutase, glutathione peroxidase and peroxiredoxin) concurrently increased (8, 43–45, 48). This suggests that redox balance may have a role in the regulation of the immune system after exercise.

Exercise is known to induce the production of reactive oxygen and nitrogen species (RONS), a diverse group of ubiquitous reactive molecules that are widely implicated in mediating cell and tissue function during exercise (11, 19, 23, 29, 30). *In vitro* evidence suggests that cell permeable RONS such as hydrogen peroxide ($H_2O_2$) can reversibly oxidise nucleophilic cysteine thiol groups that are conserved within various cellular proteins (51). These include antioxidant enzymes such as thioredoxin and peroxiredoxin (39, 52) as well as various signaling proteins, such as GTPases, phosphatases and transcription factors (33). The oxidation (i.e. sulfenylation/ sulfinylation) of thiol-containing proteins has been previously demonstrated in total lymphocyte pools following acute exercise (44, 48). This could be a potential mechanism by which $H_2O_2$ triggers redox-sensitive signaling pathways and/ or elicits oxidative stress, which may in turn influence immune cell mobilization and immunophenotype. Interestingly, recent studies have demonstrated that supplementation with the thiol donor, N-acetylcysteine (NAC) can blunt immune cell mobilization (i.e. macrophages and lymphocytes) patterns in response to both muscle-damaging (24, 31) and exhaustive aerobic exercise (28). This drives the hypothesis that shifts in thiol redox state may regulate immunoregulation.

Previous studies which have evaluated redox changes in protein thiols following acute exercise have relied on isolating all immune cells from peripheral blood (44, 48), through which downstream analysis is performed using lysates from a heterogeneous cell populations. These techniques, whilst informative, do not permit evaluation of cellular thiol redox state

within individual cells of a specific immunophenotype. Furthermore, these analytical procedures require cell lysis for their proteomic approaches, which could lead to artificial thiol oxidation, making data interpretation a challenge. Previous studies have utilized the thiol specific probe, fluorescein-5 maleimide (F5M) to label and identify intracellular proteins with solvent accessible reduced cysteines in cell line models using immunoblotting techniques (35, 53). In this respect, a loss of fluorescent signal relative to background controls is indicative of thiol oxidation i.e. an increased cellular *oxidative stress*, whereas a gain in signal would indicate *reductive stress*. Interestingly, F5M is excited at λ488 nm and emits at λ525 nm (38), offering the potential for F5M to be incorporated into flow cytometric immunophenotyping assays. Thus by adapting previous protocols that have used F5M to monitor intracellular thiol redox changes (7, 35, 53), flow cytometry would represent a powerful option to monitor *oxidative* and *reductive* stress by immunophenotype in a variety of contexts, e.g. following exercise. For example, cells of the adaptive immune system i.e. T-helper ($CD4^+$) and T-cytotoxic ($CD8^+$) lymphocytes are important to evaluate in this context, since oxidative stress has been documented in these cells post-exercise (8, 43–45, 48). Both types of T-lymphocyte are antigen specific, where $CD4^+$ T-cells serve broadly to orchestrate the immune response via cytokine production and $CD8^+$ T-cells kill infected cells through the release of cytotoxic granules (50). Whilst the importance of *oxidative stress* in $CD4^+$ and $CD8^+$ T-cells following exercise is appreciated, the role of *reductive stress* in this context is currently unknown. The impact of chronic *reductive* stress is now being appreciated in the literature (1, 20, 22), however the potential immunological significance of this response is unclear, specifically in relation to transient changes which may take place in response to exercise.

The aim of the present study was to monitor thiol-mediated redox changes in viable $CD4^+$ and $CD8^+$ T-cells isolated from individuals before and after a cycling ramp test to exhaustion. Following optimization of a flow cytometric protocol, thiol mediated redox

changes in these lymphocytes were quantified, and further studies carried out to characterise these cells with regards to immunophenotype and lymphoid homing potential.

## Materials and methods

### Participants

Following ethical approval from the University of Worcester Research Ethics Committee, twenty healthy males were initially recruited to take part in the study. Trained individuals (TR, n=9, mean ± SD: age 28 ± 5 years; BMI 23.2 ± 2.6 kg.m$^2$; $\dot{V}O_{2max}$ 56.9 ± 6.1 ml·kg$^{-1}$·min$^{-1}$) were recruited from local athletics, cycling and triathlon clubs. The criterion for "trained" was regularly completing in a minimum of 3 hours of endurance training per week, for a minimum of 2 years and a maximal oxygen consumption of at least 50 ml·kg$^{-1}$·min$^{-1}$(2). Recreationally active individuals (RA, n=11, mean ± SD: age 27 ± 6 years; BMI 24.2 ± 3.7 kg.m$^2$; $\dot{V}O_{2max}$ 45.1 ± 6.4 ml·kg$^{-1}$·min$^{-1}$) were recruited through adverts at the University of Worcester. All participants completed questionnaires addressing health history, and habitual levels of weekly physical activity (mean ± SD: TR 10235 ± 6394; RA 4932 ± 2555) and vigorous physical activity (VPA, mean ± SD: TR 800 ± 1008; RA 264 ± 271) were assessed by the International Physical Activity Questionnaire (IPAQ). Based on the results, a further six recreationally active participants (n=6, mean ± SD: age 24 ± 5 years; BMI 25.7 ± 4.1 kg.m$^2$; $\dot{V}O_{2max}$ 41.33 ± 7.63 ml·kg$^{-1}$·min$^{-1}$) were recruited to characterise the *CD8$^+$T-reduced$^+$* cell population.

All participants gave their written informed consent and the study was carried out in accordance with the Declaration of Helsinki (2008). Participants were non-smokers and reported that they had not taken any antioxidant vitamin supplements or anti-inflammatory drugs for 4 weeks prior to the laboratory visit. In addition, participants reported to be free from

any viral or bacterial infections for at least 4 weeks prior to taking part. Participants were also required to refrain from any strenuous physical activity, consumption of alcoholic beverages or caffeine for two days prior to the experimental session.

Experimental session

All experimental sessions took place at the University of Worcester in the morning (7.00-7.30am start time), following at least a 10 hour fast. After a thirty-minute period of rest, resting heart rate and blood pressure (*Omron Healthcare, Hoofddorp, The Netherlands*), height (*Seca Alpha, Hamburg, Germany*) and mass *(Tanita, Tokyo, Japan)* were determined. Cardiorespiratory fitness ($\dot{V}O_{2\,max}$) was then measured using a ramp exercise test to exhaustion on an electromagnetically braked cycle ergometer (*Lode Excalibur Sport, Groningen, Netherlands*). Workload commenced at 50 Watts and was increased by 1 Watt every 6 seconds, until volitional exhaustion or until cadence fell below 60 revolutions per minute. Oxygen uptake was assessed continuously using a breath-by-breath system (*Oxycon Prx, Jaeger, Wuerzberg, Germany*) and heart rate monitored using a Polar Vantage heart rate monitor (*Polar Vantage, Kempele, Finland*). The breath-by-breath system was calibrated daily or before each test to verify gas levels using a fixed volume cylinder (3L), a gas canister of known composition (4.5% $CO_2$, 3.5% $O_2$ and 5.0% $N_2$), as well as adjusting for any subtle atmospheric changes. A final obtained value of rate of oxygen consumption relative to body mass was accepted as $\dot{V}O_{2\,max}$ ($ml.kg^{-1}min^{-1}$) if two of the following criteria were met in conjunction with a plateau in oxygen consumption with an increase in work load: volitional exhaustion; a respiratory exchange ratio of ≥1.15; heart rate within 10 $beats \cdot min^{-1}$ of the age-predicted maximal heart rate (220 - age) (16). Upon completion of the exercise test, participants rested in a seated position for 15 minutes before the final blood sample was obtained.

Blood sampling

A catheter (*Appleton Woods, Birmingham, UK)* was inserted into the antecubital vein of the arm prior to exercise to obtain a baseline sample after thirty minutes of rest (Pre). A second blood sample was taken when participants reached predicted 70% $HR_{max}$ during the exercise test. Subsequent blood samples were taken immediately (Post+0) and 15 minutes post-exercise (Post+15). At each time point, 12 ml of blood was drawn into two separate vacutainer tubes containing potassium ethylene diaminetetraacetic acid (EDTA) (*Becton, Dickson & Company, Oxford, UK*). The catheter was flushed every 30 minutes with isotonic saline solution (0.9% sodium chloride) to prevent blood clotting. For the characterisation of $CD8^{+}T\text{-}reduced^{+}$ (n=6), two blood samples were obtained (12ml) via venepuncture to the antecubital vein of the arm prior to (Pre) and immediately following exercise (Post+0) only.

Flow cytometry method validation

Jurkat immortalised T-cell line (*ATCC, Middlesex, UK*) was maintained in RPMI-1640 adjusted to contain 10% (v/v) foetal calf serum, 2 mM L-glutamine and 100 U/ml penicillin /100 mg/ml streptomycin. Redox balance was perturbed by adding 0.1 mU/mL glucose oxidase to 2 x $10^6$ Jurkat cells (14). The cells were incubated for four hours with samples taken at the start (T0), after 1 hour (T1) and after 4 hours (T4). The samples were washed with phosphate buffered saline (PBS) and then incubated for 20 minutes with F5M (0.1 µM) in the dark at 4°C, followed by two washes with FACS buffer. The concentration of F5M was determined through titrations (0-10 µM) during pilot analysis to optimise the fluorescent signal on FL-1 FITC (data not shown). The F5M-labelled cells were subjected to flow cytometric analysis (*Guava easyCyte, Millipore UK Ltd, Hertfordshire, UK*). F5M MFI was monitored by the λ488 nm laser and λ525/30nm detector (FL1 channel). Cell viability was evaluated using trypan blue.

Blood cell isolation and sample preparation

Whole blood from each time point was used to isolate peripheral blood monocular cells (PBMCs) using density gradient centrifugation. Blood was diluted 1:1 with Hanks Balance Salt Solution (HBSS), and then layered carefully on top of Ficoll paque PLUS (*GE Healthcare, Buckinghamshire, UK*), before centrifuging at 400*g* for 40 minutes at 21°C. The PBMC layer was aspirated and then washed three times with HBSS, by centrifuging steps at 300*g* for 10 minutes.

Approximately one million cells per time point were used for flow cytometry analysis of intracellular reduced thiol content using F5M. As part of the immunophenotypic characterisation analysis, $4 \times 10^6$ /ml cells from each time point were incubated with 50 µM N-ethylmaleimide (NEM, oxidised glutathione, GSSG), and an additional $4 \times 10^6$ /ml cells with PBS (total glutathione) for 20 minutes at 4ºC to determine reduced: oxidised glutathione ratios. Cells were washed twice with FACS buffer (PBS supplemented with 2% (v/v) foetal calf serum, 0.02% sodium azide (v/v) and 5 mM EDTA) and then stored at -80 ºC.

Flow cytometry and confocal microscopy analysis

Approximately 200,000 viable PBMCs were used for identification of reduced cellular thiols in specific lymphocyte populations using four-colour flow cytometry (*Guava easyCyte, Millipore UK Ltd, Hertfordshire, UK*). Cells were incubated with fluorescently conjugated antibodies CD3-PE (clone: HIT3b), CD3-APC (clone: HIT3a) CD4-APC (clone: RPA-T4), CD8-APC (clone: HIT8a), CD8-PE (clone: SK1), CD279-PerCP (clone: EH12.2H7), CD197-PerCP (clone: G043H7) and CD28-APC (clone: CD28.2) (*Biolegend, Cambridge, UK*) for 30 minutes at 4°C followed by intermittent washes with PBS for 5 minutes at 300 x g. Cells were then incubated for 20 minutes with F5M in the dark (0.1 µM) at 4°C, followed by two washes with FACS buffer. NEM (20 µM) was used as an experimental control to confirm thiol-binding

specificity. Confocal microscopy analysis was performed to confirm that F5M had acquiesced within viable T-cells. Cells treated with F5M (0.1 μM) were stained with CD3-APC (as above). Fluorescent cells were visualised using the resonant scanning head of a TCS SP2 confocal laser microscope (*Leica, Buckinghamshire, UK*), under a x 63 oil immersion objective NA 1.32 (HCX-PL-APO). Isotype-matched control and NEM treated cells were used to determine the fluorescence threshold for the helium-neon (CD3-APC) and argon (F5M) laser respectively.

Glutathione ratio assay

Reduced: oxidised ratios of GSH:GSSG in PBMCs were quantified using a commercially available kit (*Sigma Aldrich, CS0260, Dorset, UK*), following minor modifications. Samples were prepared as described above (n=6). On the day of analysis, NEM (GSSG) and PBS-stained pellets (total glutathione) were thawed on ice and then 50 μL of Sulfosalicylic acid (5%) added to each pellet. Cells were rapidly freeze-thawed in liquid nitrogen and a 37ºC water bath twice respectively, followed by centrifugation at 10,000 *g* for 10 minutes. The supernatant was extracted and then loaded (10 μL) onto a 96-well microtiter plate with known reduced glutathione (GSH) standards (0-50 μM) in duplicate. A working solution (150 μL) of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 1.5 mg/ml) and glutathione reductase (6 units/ ml) was added to each well and left for 5 minutes at room temperature. Nicotinamide adenine dinucleotide phosphate (NADPH, 50 μL) was then added to each well, and the rate of DTNB reduction to 2-nitro-5-thiobenzoate (TNB) monitored by recording the change in absorbance every minute for 5 minutes at λ412. Nanomoles of GSH in each sample were determined for both NEM and PBS pre-incubated samples to determine GSSG and total glutathione concentrations respectively. GSH concentration was determined by subtracting GSSG from total glutathione concentration, and a subsequent ratio of GSH: GSSG used to determine half-cell reduction potential (Ehc/ mV) using the Nernst equation (36).

**Data analysis**

Flow cytometry data were analysed using GuavaSoft 3.1 (*Millipore UK Ltd, Hertfordshire, UK*). Background fluorescence was established by isotope-matched controls. Lymphocytes were identified based on forward versus side scatter and T-cells determined as being either $CD3^+$ $CD4^+$ (T-helper) or $CD3^+$ $CD8^+$ (T-cytotoxic). The CD4:CD8 ratio was determined by determining the percentage of cells within the $CD4^+$ and $CD8^+$ regions. Within each T-cell region, a distinct population of cells with high F5M fluorescence were determined (*$CD4^+$T-Reduced$^+$* and *$CD8^+$T-Reduced$^+$*). Changes in these populations were adjusted for total lymphocyte count and events within the CD3+, CD4+ and CD8+ gates and thus expressed as cells/μL. For each lymphocyte population, F5M mean fluorescence intensity (MFI) was normalised relative to pre-exercise (i.e. pre = 1), indicative of basal redox state. For the immunophenotypic characterisation analysis, lymphocytes were gated on forward versus side scatter and CD3+ $CD8^+$ cells identified. *$CD8^+$T-Reduced$^+$* cells were distinguished from *$CD8^+$T-Reduced$^-$ cells*, based on F5M binding and MFI changes in programmed cell death protein-1 (PD-1), CD28 and chemokine receptor-7 (CCR7) determined.

**Statistical analysis**

The Kolmogorov Smirnov test was used to test for normality in scale data at all time points and variables were accordingly log transformed if necessary. Differences between participant characteristics and the physiological responses to exercise were assessed using unpaired samples T-tests. The influence of training status (Trained and Recreationally active) on CD4:CD8 ratio and F5M signal were assessed over time (Pre, During, Post+0 and Post+15) by a 2*4 repeated-measures analysis of variance (ANOVA) for each of the different T-cell pools (Lymphocytes, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^+CD4^+$T-Reduced$^+$, $CD3^+CD4^+$T-

Reduced$^{-}$, $CD3^{+}CD8^{+}$T-Reduced$^{+}$ and $CD3^{+}CD8^{+}$T-Reduced$^{-}$). Post hoc analysis of any interaction effects (Group*Time) was performed by a test of simple effects by pairwise comparisons, with Bonferroni correction. GSH:GSSG ratios and MFI changes in CCR7 and CD28 over time (Pre and Post) were assessed by paired samples T-tests. MFI changes for PD-1 over time were assessed by the Wilcoxon Signed Rank Test.

All values are presented as means ± standard deviation or error (indicated throughout manuscript). Data in figures are not reported as log transformed values. Statistical significance was accepted at the $p<0.05$ level. Statistical analyses were performed using SPSS (*PASW Statistics, release 23.0, SPSS Inc., Chicago, IL, USA*).

## Results

*Flow cytometric assay optimization*

Jurkat cells exposed to 0.1 mU/ml glucose oxidase showed a F5M MFI increase from 1059 ± 203 (n=3) to 1569 ± 380 (n=3) after 1 hour (T1) compared with the start (T0). After 4 hours of incubation (T4) the F5M MFI decreased to 788 ± 211 (n=3) (Figure 1A). Normalised values ($T_n/T_0$) illustrate an increase in F5M at T1 compared to T0 and a decrease at T4 (Figure 1B). Since F5M reacts specifically with reduced thiols, these data indicate *reductive* stress at T1 and oxidative stress at T4 (Figure 1B). Treatment with glucose oxidase had no overall impact on cell viability (data not shown).

*Physiological responses to the exercise test*

To assess the physiological responses of TR and RA groups to exercise, $\dot{V}O_{2\,max}$, power output, heart rate and test duration were measured and reported in Table 1. All participants achieved $\dot{V}O_{2\,max}$ with regards to our criteria. TR had a significantly greater $\dot{V}O_{2\,max}$ ($p=0.001$)

and maximal power output relative to body mass (p=0.003) than RA. No significant differences in test duration, timing of the blood sample during exercise or peak heart rate were noted between TR and RA. There were no statistically significant differences in the physiological responses between the two RA cohorts when characterising lymphocyte thiols (n=20) and subsequent immunophenotyping (n=6).

*Analysis of the lymphocyte composition of peripheral blood in response to exercise*

Changes in the ratios of $CD4^+$ to $CD8^+$ T-cells are reported in Figure 2. A significant reduction in CD4:CD8 ratio was observed during and immediately post-exercise only in RA (Figure 2, Group*Time effect: $F_{3,54} = 3.77$, p=0.016). The ratio decreased at all time points relative to Pre (p<0.0001), and was lower at Post+0 relative to During (p<0.0001) in RA. In TR, the ratio was significantly lower only at Post+0 relative to Pre (p=0.004). The ratio increased Post+15, relative to Post+0 (TR p=0.045; RA p=0.041) in all participants, but was only significantly lower than Pre in RA (p<0.0001). At rest, CD4:CD8 ratio was higher in RA, relative to TR (p=0.016).

*Analysis of intracellular reduced thiol content in lymphocytes by immunophenotype*

Following this, we applied our flow cytometry method to determined intracellular *reduced* cysteine thiol fluorescence in viable human T-populations (Figure 3) in response to exercise (Figures 4 and 5). Using a hierarchical gating strategy, it was first determined that exercise caused an increase in the normalised F5M ratio at Post+0 in gated lymphocyte (time effect: $F_{3,54} = 4.2$, p=0.010) and $CD3^+$ pools (time effect: $F_{3,54} = 4.1$, p=0.011) in all participants. These changes were driven by $CD8^+$ (time effect: $F_{3,54} = 3.5$, p=0.029 – Figure 5A) and not

$CD4^+$ T-cells (time effect: $F_{3,54} = 2.0$, p=0.132 – Figure 4A). There were no differences in F5M normalised ratios between RA and TR groups in either T-cell population.

Alterations in the *$CD4^+$T-Reduced$^+$* and *$CD8^+$T-Reduced$^+$* concentration are reported in Figures 4C and 5C. A significant increase in the concentration of *$CD8^+$T-Reduced$^+$* cells at Post+0, relative to Pre (p=0.003) and During (p=0.009) were noted in recreationally active participants only (time*group interaction effect: $F_{3.54} = 3.821$, p=0.015). At Post+15, the concentration of *$CD8^+$T-Reduced$^+$* cells was not greater than Pre (p=0.216). No differences were noted in the concentration of *$CD4^+$T-Reduced$^+$* cells in either group.

*Characterization of $CD8^+$T-Reduced$^+$*

To further understand these observations, immunophenotypic characterization of the CD*$8^+$T-Reduced$^+$* cells was carried out in a different sample of 6 individuals. Markers of T-cell activation (CD28), suppression (PD-1) and lymphoid homing (CCR7) were stratified according to the F5M signal before and after exercise. MFI changes in CD28, PD-1 and CCR7 are reported in Figure 6, with the exercise test data for this sample reported in Table 2. A significant decrease in CCR7 MFI was observed immediately after exercise in *$CD8^+$T-Reduced$^-$* (*$CD8^+$T-Reduced$^+$*: p=0.173; *$CD8^+$T-Reduced$^-$*: p=0.027). However, *$CD8^+$T-Reduced$^+$* exhibited a significantly lower CCR7 MFI relative to *$CD8^+$T-Reduced$^-$* after exercise (p=0.042). PD-1 expression in gated $CD8^+$ cells was unchanged in response to exercise (*$CD8^+$T-Reduced$^+$*: p=0.655; *$CD8^+$T-Reduced$^-$*: p=0.273). PD-1 expression was lower in *$CD8^+$T-Reduced$^+$*, compared to *$CD8^+$T-Reduced$^-$* at both time points but did not reach statistical significance (Pre-Exercise: p=0.068; Post-Exercise: p=0.109). No differences were noted in CD28 expression between the two populations.

*Glutathione analysis*

To identify a redox couple driving the observed *reductive* shift in cellular redox state, changes in the most abundant cellular redox couple, GSH:GSSG, was first quantified. The concentrations of GSH and GSSG were used to calculate an Ehc/ mV for the pool of isolated PBMCs using Nernst equations (36). No difference in PBMC Ehc/ mV was observed in response to exercise (pre: -209 ± 20 to post: –206 ± 16. Ehc/ mV).

## Discussion

The present study represents the first to investigate changes in lymphocyte solvent accessible reduced thiols in response to exhaustive exercise in healthy young males. Using the thiol-specific and fluorescent probe, F5M, we identified that the lymphocyte pool becomes transiently *reductive* in response to exercise. Within the lymphocyte pool, $CD8^+$ T-cells demonstrated a greater F5M fluorescence than $CD4^+$ T-cells immediately after exercise (Figures 4 and 5). At this time point, a population of $CD8^+$ T-cells was identified with high F5M binding capacity ($CD8^+$*T-reduced*$^+$), and it was demonstrated that the concentration of these cells was greater in recreationally active than aerobically trained participants. We carried out further analysis to characterise immunophenotype specific exercise-induced changes in $CD8^+$*T-reduced*$^+$ in a separate group of recreationally active individuals. Collectively, the present data suggests that immediately after a single bout of exercise, peripheral blood is composed of more $CD8^+$*T-reduced*$^+$ cells that have a lower expression of CCR7, relative to $CD8^+$*T-reduced*$^-$.

It is well documented that exercise of sufficient intensity and duration can induce a transient increase in markers of *oxidative stress* (42, 43). As a result, multiple studies have reported elevated indices of oxidative stress following exercise in skeletal muscle (34, 46), blood cells

(erythrocytes (3) and leukocytes(43, 48)) and extracellular fluids (plasma (49), saliva (6) and urine (37, 40)). The results of our study using an optimised flow cytometric approach provide evidence that the immune cell compartment of peripheral blood undergoes transient *reductive stress* after exercise. Of the 20 participants studied, 17 demonstrated a reductive shift in $CD8^+$ cell thiol redox state either immediately or 15 minutes after exercise (Figure 5). To our knowledge, reports of cellular *reductive stress* in humans have only been observed in chronic neurological (1, 20) and cardiovascular disease (25). Margaritelis et al (22) made the observation that many studies reporting mean trends in a specific extracellular marker of oxidative stress dismissed potential 'reductive' outliers as part of the dataset. In support of this, the present data supports the notion of acute intracellular *reductive stress* after exercise.

Determining the role of protein thiols as regulators of exercise modified redox balance is an evolving area of investigation (5). *Reductive stress* implies increased electron availability within the cell or a subcellular compartment whereby an abundant redox couple accepts more electrons. Although less abundant, signaling protein thiols may also become reduced in this context, altering key signaling pathways after exercise (47). Indeed, previous evidence has shown that the increased availability of low molecular weight *reduced* protein thiols such as Glutathione (GSH) within T-cells facilitates their proliferation and secretion of Interleukin-2 (13). We quantified GSH:GSSG ratio to estimate changes in global thiol redox balance within PBMC lysates in response to exercise. Using Nernst equations based on Schafer et al (36), no changes were observed in PBMC mV after exercise (pre: -209 ± 20 to post: –206 ± 16. Ehc/ mV). We performed further analysis as part of a proof of concept approach to label $CD8^+$ T-cells with F5M and assess exercise-induced changes in solvent accessible cysteine thiol redox state, using mass spectrometry (see supplementary information). These preliminary data indicate that specific proteins can be identified (e.g. including transcription factors), and alterations in thiol redox state interpreted after exercise. Further analysis is required to identify

the redox couples involved in the *reductive* shift in $CD8^+$ T-cells. It must be emphasized that the results of the current study do not definitively pinpoint whether increased $CD8^+$*T-reduced*$^+$ cells are a result of cysteine thiol mobilisation or shifts in $CD8^+$ cell immunophenotype. The former would imply higher intracellular reductive capacity that may result from an altered flux of glucose through the pentose phosphate pathway during exercise (41), thus increasing the cellular pool of NADPH, a cofactor for thiol reducing enzymes such as thioredoxin reductase (15). With regards to the immunophenotype, there is evidence to suggest that late differentiated $CD8^+$ T-cells have higher basal thiol concentration than naive $CD8^+$ T-cells (45). Late differentiated CD8+ T-cells have high effector function and are known to be highly responsive to exercise-induced mobilization (4). This would suggest that an increase in $CD8^+$*T-reduced*$^+$ cells could relate to an altered composition of CD8+ T-cells, rather than altered redox balance intracellularly. Only extensive immunophenotypic and functional analysis of $CD8^+$ cells over a suitable time course would be able to determine the origin of the observed *reductive stress* in the immune cell compartment of peripheral blood.

The current data supports previous work showing an elevation in $CD8^+$ T-cells in peripheral blood during and in the minutes following exercise (4), with the relative increase in $CD8^+$ cells exaggerated in individuals with lower training status (Figure 2). The CD4:CD8 ratio decreased During (70% $HR_{max}$) and Post+0 in RA, but only Post+0 in TR. This indicates that RA participants were more sensitive to $CD8^+$ cell mobilization. In conjunction with this data, a novel finding herein was the identification of a population of $CD8^+$ T-cells with a high capacity to bind F5M immediately post-exercise ($CD8^+$*T-reduced*$^+$, Figure 5B). The concentration of $CD8^+$*T-reduced*$^+$ cells present Post+0 increased from pre-exercise values in RA, with no change noted in TR individuals (Figure 5C). As a proportion of the CD8+ pool, $CD8^+$*T-reduced*$^+$ cells increased from 16 to 42% post-exercise in RA, with a 4% decrease noted in TR (data not shown). No differences were noted in $CD4^+$*T-reduced*$^+$ concentration or proportional

composition between groups or in response to exercise (Figure 4C), suggesting that the *reductive* shift observed in $CD8^+$ thiol redox state may relate to the mobilization patterns of immune cells in response to exercise.

To our knowledge, transient differences in intracellular redox state following exercise, based on training status have not been previously reported. However, there is evidence to suggest that physical training can upregulate the expression of endogenous antioxidant enzymes in both skeletal muscle (12, 18) and immune cells (42, 43). We can therefore hypothesize that enhanced antioxidant protection at rest may have therefore provided improved tolerance to exercise-induced ROS in the TR group and reduced the need for these individuals to mobilise $CD8^+$*T-reduced*$^+$ cells immediately after exercise. It must be noted that there were no statistical differences in total MFI changes in F5M between groups at Post+0, indicating that $CD8^+$ cells in TR were also transiently *reductive*, however these individuals did not exhibit exercise-induced changes in $CD8^+$*T-reduced*$^+$ cell concentration. With regards to $CD8^+$*T-reduced*$^+$ cells, no correlation was noted between the concentration of $CD8^+$*T-reduced*$^+$ and $CD8^+$ cells present in the circulation at Post+0 in RA (*data not shown*), suggesting no direct relationship between $CD8^+$ mobilization and thiol redox state; however, as mentioned above, this may be due to the precise immunophenotype of the $CD8^+$ cells not being fully characterized. Nevertheless, our findings strengthen the growing body of evidence that suggests that *reduced* protein thiols may play an important role in the mobilization of specific subsets of immune cells after exercise, with training status of the individual being a factor worthy of further investigation.

Based on the above findings, an additional six recreationally active participants were recruited in order to characterize the immunophenotype of $CD8^+$*T-reduced*$^+$ cells in response to the same exercise protocol. We coupled F5M and antibody staining to compare alterations in thiol redox state with changes in cell markers of T-cell: activation (CD28), suppression (PD-

1) and lymphoid homing (CCR7) before and immediately after exercise (Figure 6: B-D). CCR7 expression facilitates the migration of T-cells to secondary lymphoid organs i.e. lymph nodes, where interaction with other immune cells takes place. This process plays a central role in immune cell activation and clonal proliferation of antigen specific T-cells (9). A significant decrease in CCR7 expression was noted in $CD8^{+}$*T-reduced*$^{-}$ cells after exercise only (Figure 6C). Interestingly, $CD8^{+}$*T-reduced*$^{+}$ cells expressed lower levels of CCR7 than $CD8^{+}$*T-reduced*$^{-}$ cells post exercise. CCR7 is a lymphoid homing receptor expressed on naïve T-cells located in the primary lymphoid organs (i.e. thymus and bone marrow). Lower expression of CCR7 is known to mediate trafficking of effector T-cells towards secondary lymphoid organs (32). Therefore, given that $CD8^{+}$*T-reduced*$^{+}$ have less lymphoid homing capacity, but may be mobilised to a greater extent during exercise, these findings propose a role for redox-mediated driven immune cell mobilization and possibly, extravasation. No changes in CD28 expression were noted, suggesting that acute cellular *reduction* has little impact on co-stimulatory marker expression (Figure 6D). A trend for lower expression levels of the immunosuppressive cell-surface receptor, PD-1 was observed in $CD8^{+}$*T-reduced*$^{+}$ compared to $CD8^{+}$*T-reduced*$^{-}$ cells ($p=0.068$, Figure 6B). PD-1 is a cell surface receptor expressed on activated T-cells that downregulates antigen-mediated T-cell activation. Interestingly, elevated PD-1 expression has been observed in many cancers (17, 21, 27), with subsequent T-cell exhaustion promoting poor anti-tumor responses.

We have validated a flow cytometry method *in vitro* (Figure 1), indicating that a standard physiological dose of enzyme-mediated $H_2O_2$ can elicit *oxidative* and *reductive* stress in Jurkat cells. This method has high utility for determining the behavior patterns of specific immune cell populations in response to exercise in humans. Further research is needed to expand on our findings regarding CCR7 and PD-1; to more accurately characterise the relationship between T-cell function, thiol redox state and the physiological relevance to exercise and immune

function. Given that thiol labeling is achieved prior to cell lysis, advanced proteomic analysis can be used to determine modifications to solvent accessible thiol proteins that may alter cellular function (see supplementary information).

We must acknowledge some potential limitations to the present study. The application of F5M in highly diverse biological samples such as human PBMCS is subject to some subtle differences in membrane permeability, cross-reactivity with other nucleophiles (e.g. with primary and secondary amines) and probe hydrolysis, the latter two of which are pH dependent (pH>7.5). Decreases in lymphocyte pH (7.4) following a $\dot{V}O_{2max}$ test would likely be minimal (10, 26), however, whereby our method is applied to more prolonged exercise models, subtle changes in pH should be accounted for. In line with this comment, it must also be noted that our results only reflect immune thiol redox state changes in response to a $\dot{V}O_{2max}$ test, and not bouts of exercise that are more prolonged and sustained at moderate or high intensity. Future work is needed to clarify our current findings in response to more conventional types of exercise.

## Conclusion

The present study provides evidence that the peripheral blood lymphocyte pool becomes transiently more *reductive* in response to acute exhaustive exercise in healthy males. Using the fluorescent probe, F5M we have identified a specific population of $CD8^+$ T-cells ($CD8^+$*T-reduced*$^+$) that drives this reductive shift, with training status appearing to be a key variable differentiating individuals. We provide evidence to suggest that $CD8^+$*T-reduced*$^+$ may target secondary lymphoid organs post exercise and recommend that further research is undertaken to validate these observations, and characterise the significance of *oxidative* and *reductive* shifts in immune cell redox state post exercise.

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**Conflict of Interest**

None of the authors declare a conflict of interest.

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**Tables**

*Table 1:* **Exercise physiology data *(n=20)***: test duration, relative peak power output (Watt/ kg), peak heart rate (bpm) and relative maximal oxygen consumption achieved during the exercise test. All values are means (standard deviation).

| | **Trained** (n=9) | **Recreationally Active** (n=11) |
|---|---|---|
| **Test Duration** (min:sec) | 27:31 (07:03) | 22:44 (06:04) |
| **Timing of During Exercise Sample** (min:sec) | 14:03 (03:35) | 12:27 (04:06) |
| **Relative Watt Max** ($Watt \cdot kg^{-1}$) | 4.24 (0.48) $^{\$\$}$ | 3.45 (0.53) |
| **Peak Heart Rate** ($beats \cdot min^{-1}$) | 185.89 (11.76) | 189.36 (5.85) |
| **Relative $VO_2$max** ($ml \cdot kg^{-1} \cdot min^{-1}$) | 56.93 (6.09) $^{\$}$ | 45.10 (6.44) |

*Table 1 Legend:* $^{\$}$ indicates a significant difference between trained and recreationally active groups (Group effect): $^{\$}$ $p<0.05$; $^{\$\$}$ $p<0.001$.

*Table 2:* **Participant demographics and physiological data from participants taking part in the immunophenotyping analysis arm of the study (n=6):** Values are means (standard deviation).

| | n=6 |
|---|---|
| **Test Duration** (min:sec) | 18:07 (03:02) |
| **Relative Watt Max** ($Watt \cdot kg^{-1}$) | 2.98 (0.60) |
| **Peak Heart Rate** ($beats \cdot min^{-1}$ | 180.5 (9.67) |

| | |
|---|---|
| **Relative $VO_{2max}$** ($ml \cdot kg^{-1} \cdot min^{-1}$) | 41.33 (7.63) |

***Fig 1:*** **Optimisation of the F5M flow cytometric assay using Jurkat cells (*n=3*). (A)** Representative flow cytometric plot illustrating Jurkat cells labelled with F5M following glucose oxidase treatment. T0 = start, T1 = 1 hour incubation with glucose oxidase and T4 = 4 hours incubation with glucose oxidase. Intracellular thiol redox status was measured by F5M binding and a change in FL1-H (λ488nm; λ525/30nm) intensity. A cell sample unstained with F5M is also represented. **(B)** Summarised data illustrating normalised F5M signal ($T_n/T_0$). Indicated is 'reduction' i.e. more free cys-thiol relative to T0 and 'oxidation' i.e. less free cys-thiol relative to T0.

***Fig 2:*** **Changes in T-cell populations in response to acute exercise (*n=20*).** Bars represent CD4:CD8 ratio changes in RA (grey bars) and TR (white bars) Pre, During, Post+0 and Post+15. Values are means ± standard deviation. * indicates a significant difference relative to Pre: * $p<0.05$, *** $p<0.0001$. $^{+}$ indicates a significant difference relative to During: +++ $p<0.0001$. $^{\sharp}$ indicates a significant difference relative to Post: $^{\sharp}$ $p<0.05$. $^{\$}$ indicates a significant difference between RA and TR (all Time*Group effects; pairwise comparisons).

***Fig 3*** **Immunophenotypic assessment of reduced intracellular thiol content in viable T-cell populations (*n=20*). (A)** Representative gating strategy for $CD8^+$ cells. Lymphocytes were gated (left panel) on forward light scatter (FSC) *vs.* side light scatter (SSC) and then stained for CD3+ (PE) and $CD8^+$ (APC, right panel). **(B)** Intracellular reduced thiols were measured by a gain of F5M binding and increase in FL1-H (λ488nm; λ525/30nm) intensity. Unstained and an experimental control (NEM) were used to confirm thiol binding specificity **(C)** A

representative PBMC confocal image illustrating intracellular F5M signal (left image) and surface staining for T-cells with anti-human CD3-APC (right image).

***Fig 4:*** **Flow cytometric analysis of intracellular thiol redox status in $CD4^+$ cells in response to acute exercise (*n=20*). (A)** Bars represent normalised MFI on FL1-H (λ488nm; λ525/30nm) in gated $CD4^+$ cells for RA (grey bars) and TR (white bars) participants. Data are normalised relative to pre-exercise fluorescence **(B)** Panel shows a representative overlaid FL1 histogram of $CD4^+$ cells Pre (grey fill) and Post+0 (white fill) treated with 0.1 μM F5M. *$CD4^+$T-Reduced$^+$* was identified based on a highly reduced population of cells on the far right of the histogram **(C)** Bars represent changes in the concentration of *$CD4^+$T-Reduced$^+$* cells per μL in RA (grey bars) and TR (white bars) participants in response to acute exercise. All values are means ± standard error.

***Fig 5:*** **Flow cytometric analysis of intracellular thiol redox status in $CD8^+$ cells in response to acute exercise (*n=20*). (A)** Bars represent normalised MFI on FL1-H (λ488nm; λ525/30nm) in gated $CD8^+$ cells for RA (grey bars) and TR (white bars) participants. Data are normalised relative to pre-exercise fluorescence **(B)** Panel shows a representative overlaid FL1 histogram of $CD8^+$ cells Pre (grey fill) and Post+0 (white fill) treated with 0.1 μM F5M. *$CD8^+$T-Reduced$^+$* was identified based on a highly reduced population of cells on the far right of the histogram **(C)** Bars represent changes in the concentration of *$CD8^+$T-Reduced$^+$* cells per μL in RA (grey bars) and TR (white bars) participants in response to acute exercise. All values are means ± standard error. * indicates a significant difference relative to Pre: ** $p<0.001$. $^+$ indicates a significant difference relative to During: ++ $p<0.001$ (all Time*Group effect; pairwise comparisons).

***Fig 6:*** **Immunophenotypic analysis of *CD8$^+$T-Reduced$^+$* activation, suppression and lymphoid homing following exhaustive exercise (*n=6)*. (A)** Chart illustrates changes in MFI for F5M in response to exercise **(B-D)** Bars represent MFI for PD-1 (B), CCR7 (C) and CD28 in *CD8$^+$T-Reduced$^+$* (white bars) and *CD8$^+$T-Reduced$^-$* (black bars) before and after exercise. Values are means ± standard deviation. * indicates a significant difference relative to Pre (Time*Group effect; pairwise comparison): * $p<0.05$. $^{\#}$ indicates a significant difference between *CD8$^+$T-Reduced$^+$* and *CD8$^+$T-Reduced$^-$* (Time*Group effect; pairwise comparison): $^{\#}$ $p<0.05$.

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